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Establishment of Pregnancy in the Pig: I. Interrelationships Between Preimplantation Development of the Pig Blastocyst and Uterine Endometrial Secretions[1,2]

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ABSTRACT

Pregnancy recognition in pigs occurs between Days 11 and 12 when blastocysts undergo transformation from the spherical to filamentous form. This study evaluated the relationship between blastocyst development and total calcium (Ca), estrone (E_1), estradiol (E_2), estriol (E_3), E_1 sulfate (E_1S), E_2S, E_3S, prostaglandin F (PGF), PGE_2, protein (Pr) and acid phosphatase (AP) activity in uterine flushings obtained from pregnant gilts between Days 10 and 14. Data from pregnant gilts were compared to those obtained from uterine flushings collected between Days 10 and 14 of the estrous cycle. Surface and ultrastructural changes in the endometrium associated with blastocyst development were evaluated by scanning (SEM) and transmission (TEM) electron microscopy. Pregnant uterine flushings were analyzed relative to average size of blastocysts recovered: 5 mm spherical, 5–8 mm spherical, 9–50 mm tubular, and >50 mm filamentous Day 12 and Day 14. Nonpregnant gilt uterine flushings were analyzed by Day of the estrous cycle (10.5, 11, 11.5, 12 and 14). Total E_2 content increased almost 4-fold in pregnant uterine flushings containing tubular blastocysts (2.3 ng) as compared with flushings with spherical blastocysts (0.6 ng) and continued to increase as the blastocysts became filamentous (4.4 ng) but had declined by Day 14 (0.4 ng). The pattern of change for total recoverable E_1 and E_2 was similar to that for E_2 and the highest values were obtained in uterine flushings with filamentous blastocysts. A concomitant increase in E_1S and E_2S content also was detected in flushings with tubular and Day 12 filamentous blastocysts. Total Ca, Pr, AP, PGF, and PGE_2 content increased in association with increased E_2 in flushings. The increase in Pr, AP, PGF and PGE_2 content in pregnant flushings continued to Day 14. However, Ca content had declined by Day 14 (0.1 mg) after a transient increase to 1.5 mg in flushings that contained tubular blastocysts (Days 11 and 12). Comparable changes in estrogens, proteins, Ca, PGF and PGE_2 content in nonpregnant uterine flushings collected between Days 10.5 and 14 were not detected. Electrophoresis of protein in pregnant uterine flushings indicated that the appearance of three basic uterine proteins (M_r 32K to 60K) were associated with the increase in estrogen. These proteins were detected in flushings with tubular and filamentous blastocysts (Day 12), but were not found until Day 14 in nonpregnant gilts. A synchronized release of secretory vesicles from the glandular epithelium was observed by TEM which indicated a close association between formation of tubular blastocysts, onset of blastocyst estrogen production and increased protein in uterine flushings. Although secretion was detected in nonpregnant glandular epithelium, no synchronized release was observed. Results suggest that estrogen production by tubular and early filamentous blastocysts (Day 11.5–12.0) stimulates secretion of protein from the endometrium which may be mediated through an effect of free Ca on the uterine glandular epithelium. Increases in PGF and PGE_2 were associated closely with estrogen production and blastocyst elongation.

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INTRODUCTION

Pig blastocysts undergo marked morphological change between Days 10 and 16 of pregnancy. The most rapid period of blastocyst elongation is between Days 10 and 12 when changes from spherical (3 to 10 mm diameter) to tubular (10 to 50 mm long) to filamentous (>100 mm long) forms occur, attaining lengths

of 700 to 1000 mm by Day 16 of pregnancy (Perry and Rowlands, 1962; Anderson, 1978).

Two critical events are associated with blastocyst elongation between Days 10 and 12 of pregnancy. First, estrogen production by blastocysts is initiated during this period (Perry et al., 1973; Perry et al., 1976; Flint et al., 1979; Gadsby et al., 1980; Heap et al., 1981) and these estrogens appear to be the blastocyst "signal" for maternal recognition of pregnancy in swine (Bazer and Thatcher, 1977; Flint et al., 1979). The second event is associated with blastocyst elongation and leads to sequestering of histotroph, i.e., endometrial epithelial cell secretions in the uterine lumen to provide nourishment for developing blastocysts (Bazer and Thatcher, 1977; Zavy et al., 1980). Pig blastocysts do not initiate complete attachment to the endometrial epithelium until Day 18 of gestation (Crombie, 1972); therefore, availability of soluble nutrients within the uterine lumen is assumed to be critical to embryonic survival.

Aitken (1977) detected a rapid increase in total unconjugated estrogens in plasma of roe deer during the period of blastocyst elongation. Coincident with this event, there was a concomitant increase in uterine luminal protein, carbohydrate, calcium and amino nitrogen. No increase in conjugated (Robertson and King, 1974) or unconjugated (Guthrie et al., 1972) estrogens has been detected in peripheral plasma of pregnant pigs between Days 10 and 12; however, estradiol and/or estrone concentrations were reported to increase in uteroovarian vein plasma (Moeljono et al., 1977) and in uterine flushings (Zavy et al., 1980) during that period.

The present study was designed to: *1)* determine the precise temporal relationship between stage of blastocyst development, i.e., spherical, tubular and filamentous stages, and initiation of estrogen production and *2)* determine the relationship between initiation of estrogen production by blastocysts and onset of uterine secretory activity in gilts between Days 10 and 14 of pregnancy.

MATERIALS AND METHODS

General

Thirty-six sexually mature crossbred gilts of similar age (7.5–8.5 months), weight (105 to 115 kg), and genetic background were utilized in this study after having experienced two estrous cycles of normal duration (18 to 22 days). Gilts were observed for estrus twice daily in the presence of intact boars and day of onset of estrous behavior was designated Day 0. Gilts assigned to be mated were bred at the time of detection of estrus and at 12 h and 24 h after detection of estrus.

In a preliminary experiment, uterine flushings (n = 10) were obtained from bilaterally hysterectomized nonpregnant and pregnant gilts on Days 10.5, 11.0, 11.5, 12.0 and 14.

In the major experiment, 11 nonpregnant and 15 pregnant gilts were hysterectomized unilaterally on Day 10.5 after detection of estrus. This allowed collection of uterine flushings from pregnant gilts having spherical blastocysts (3 to 7 mm diameter) and nonpregnant gilts at a comparable period after onset of estrus. The second uterine horn was removed from nonpregnant gilts on either Day 11, 11.5 or 12. For pregnant gilts, the second uterine horn was removed between Days 10.5 and 12 or between 6 and 36 h after removal of the first uterine horn. Surgical procedures were those described by Murray et al. (1972). Uterine flushings were obtained according to the procedure of Zavy et al. (1980) and pregnancy was confirmed in bred gilts by the presence of morphologically normal blastocysts.

Uterine flushings obtained as described previously (Murray et al., 1972; Zavy et al., 1980) were centrifuged at 10,000 × *g* for 20 min and supernatant stored at −20°C until analyzed. Sections of uterine endometrium were obtained immediately after collection of the uterine flushing and fixed for scanning and transmission electron microscopy as described later.

Hormonal Analyses

Uterine flushings from pregnant and nonpregnant gilts were analyzed for concentrations of estrone (E_1), estradiol (E_2), estriol (E_3), estrone sulfate (E_1S), estradiol sulfate (E_2S), estriol sulfate (E_3S), immunoreactive prostaglandin F (PGF) and prostaglandin E_2 (PGE_2).

Radioimmunoassays (RIA) for E_1 and E_2 in uterine flushings were validated previously in our laboratory (Zavy et al., 1980). Free estrogens (E_1, E_2 and E_3) were extracted twice with diethylether and separated by Sephadex LH-20 column chromatography as previously described for cow (Chenault et al., 1975; Eley et al., 1979) and pig (Knight et al., 1977) plasma. An antibody to E_2 was used for determination of both E_1 and E_2 concentrations (gift from Dr. V. L. Estergreen of Washington State University). The sensitivity of the assay was 10 pg/ml and intra- and inter-assay coefficients of variation for E_1 were 4.4% and 19.5% and for E_2, 22.7% and 22.1%. Average recovery of the radiolabeled internal standards was 87%, 75% and 68% for E_1, E_2 and E_3, respectively.

Estriol concentrations were measured by RIA after column chromatography. A specific antibody to E_3, donated by Dr. Pushpa Kalra (University of Florida, Gainesville) was used. Cross-reactivity of this antibody was 100% for E_3, 22% for 17β-E_2, 6.6% for E_1 and 0.5% or less for 17α-E_2, P_4, DHT and DHEA. After isolation by Sephadex LH-20 column chromatography, E_3 was quantified using an antibody dilution of 1:7,000 giving an assay sensitivity of 10 pg. The E_3 assay was validated by addition of known amounts of unlabeled E_3 (100 to 1,000 pg) to 2 ml of uterine flushings from a Day 10 nonpregnant gilt. There was a

linear relationship between E_3 added and amount measured in uterine flushings (Y = 29.0621 + 1.0740 X; R^2 = 0.99) The intraassay coefficient of variation was 7.2% for the validation.

Determination of conjugated estrogens in pig uterine flushings was according to procedures previously described for cow plasma (Eley et al., 1981). Two-milliliter aliquots of each uterine flushing were extracted with freshly distilled benzene. Uterine flushings containing conjugated steroids were then submitted to enzymatic hydrolysis following extraction of the unconjugated phenolic steroids. Hydrolysis of uterine flushings was accomplished by addition of 10 units Helix pomatia Type H-2 enzyme (Sigma Chemical Co., St. Louis, MO) in 5 ml of 0.1 M acetate buffer (pH 4.0). This enzyme preparation contains both sulfatase and B-glucuronidase activities. After overnight incubation at 37°C, the reaction was terminated by adding 2.5 ml of 0.2 M Tris buffer (pH 10.5). The liberated estrogens were then treated as free estrogens with subsequent diethylether extraction, column chromatography and RIA for E_1, E_2 and E_3. Assay for conjugated estrogens was validated by addition of known amounts of estrone sulfate (100 to 1,000 pg) to 2 ml of uterine flushings from a Day 10 nonpregnant gilt. A linear relationship between E_1S added and measured in uterine flushings was detected (Y = 74.8575 + 0.8506 X; R^2 = 0.96). The intra- and inter-assay coefficients of variation were 24.4 and 16.7%, respectively. The sample values were corrected for procedural losses and represent free E_1, E_2 and E_3 values multiplied by 1.4. Recovery of internal standards for E_1S, E_2S and E_3S were 75%, 64% and 65%, respectively. Intra- and inter-assay coefficients of variation were 24% and 16% for E_1S, 12% and 5% for E_2S and 24% and 24% for E_3S.

The PGF assay, previously validated in our laboratory, for porcine uterine flushings (Zavy et al., 1980) was used for measurement of PGF concentrations in all uterine flushings. Cornette et al. (1972) indicated that the cross-reactivities of PGF antibody with other prostaglandins were: <0.1% for PGA, PGA_2 PGB, PGB_2, PGE_2, PGE_3; 1% for 15-keto PGF_2 and 8% for PGF_1. Therefore, data are expressed as immunoreactive PGF and not $PGF_2\alpha$. Samples were measured in a single assay with an intraassay coefficient of variation of 15%.

Determination of prostaglandin E_2 concentrations in uterine flushings was based on the method described for sheep plasma (Lewis et al., 1978). After addition of radiolabeled (5, 6, 8, 11, 12, 14, 15 [^{3}H]PGE_2; New England Nuclear, Boston, MA) recovery marker, 0.1 ml of 1 N HCl as added to 1 ml of uterine flushing followed by extracting twice with freshly distilled ethyl acetate. Ethyl acetate was evaporated under nitrogen and Sephadex LH-20 column chromatography by using a solvent system of 98% methylene chloride and 2% methanol to isolate PGE_2. The PGE_2 antibody used in the RIA was a gift from Dr. N. R. Mason of Lilly Research Laboratories, Indianapolis, IN. Cross-reactivities of the antibody (Lewis et al., 1978) with other prostaglandins were: 16.8% for PGE_1, 2.8% for PGA_2; 0.5% for PGA_1, $PGF_2\alpha$, 15-keto PGE_2 and 13,14 dihydro-15-keto PGE_2. A 1:6000 dilution of antiserum was used after isolation of PGE_2 giving an assay sensitivity of 10 pg. When known amounts of PGE_2 (50, 100, 250, 500, 1000 and 2500 pg) were added to 1 ml of uterine flushing from a Day 10 nonpregnant gilt, a linear relationship between PGE_2 added and that measured in uterine flushings was obtained. (Y = 31.2199 + 1.2580 X; R^2 = 0.99). The intraassay coefficient of variation for the validation was 12%. Samples were measured in a single assay with an intraassay coefficient of variation of 19%.

Analysis of Protein and Acid Phosphatase Activity

Protein concentration in uterine flushings was determined by the method of Lowry et al. (1951). Acid phosphatase activity was determined, using *p*-nitrophenyl phosphate as substrate, as previously described by Shlosnagle et al. (1976) and Basha et al. (1979). One unit of activity was defined as the capacity to release 1 μmole nitrophenol per minute at pH 4.9 in 0.1 M acetate buffer.

Analyses of Calcium

Calcium concentrations were measured with the Calcett manufactured by Precision Systems, Inc. (Sudbury, MA). This system utilized EGTA for fluorometric titration of calcium in aqueous solutions (Alexander, 1971).

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Uterine flushings (2 mg protein) were dialyzed (mol. wt cut-off ≅ 3500) against 1 mM Tris-HCl buffer (pH 8.2) and then lyophilized. After lophilization, the dialyzed uterine protein was prepared for 2D-PAGE by directly dissolving it in 1 ml of 5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) nonidet P-40 and 0.5% (w/v) dithiothreitol (Horst and Roberts, 1979). The 2D-PAGE was performed using 300 μg protein for acidic and basic uterine proteins according to modification of the method of O'Farrell (1975) as described by Horst and Roberts (1979), Horst et al. (1980) and Basha et al. (1980). Standard proteins for obtaining molecular weight estimates were bovine serum albumin, ovalbumin, aldolase, pepsin, chymotrypsinogen A and cytochrome C prepared as described for the samples. Determination of pH gradients was by the method of Horst and Roberts (1979). No differences in 2D-PAGE protein profiles have been observed between samples centrifuged prior to processing at 10,000 × *g* for 20 min, as in the present study, versus 100,000 × *g* for 60 min (R. M. Roberts, unpublished data).

Scanning (SEM) and Transmission (TEM) Electron Microscopy

Immediately after obtaining uterine flushings, endometrium was removed, cut into 1–2 mm cubed sections, and immersed in a fixative containing 2.0% glutaraldehyde and 0.01 M sucrose in 0.1 M cacodylate buffer (pH 7.2). Tissue was rinsed (3×) with 0.1 M cacodylate buffer followed by osmication for 1 h in 1% OsO_4. Specimens used for scanning electron microscopy (SEM) were dehydrated in a graded series of alcohols (25, 50, 75, 100 and 100%) followed by critical point drying in CO_2. Tissue was mounted on stubs with low resistance contact cement (E. F. Fullman, Schenectady, NY), sputter-coated with gold

and viewed on a Hitachi S-450 electron microscope operating at 20KV.

Endometrium for transmission electron microscopy (TEM) was fixed additionally in 1% tannic acid with en bloc staining with uranyl acetate after osmication. Dehydration was in graded alcohols (25, 50, 75, 100 and 100%) followed by 100% acetone. Tissue sections were then embedded in Spurr's plastic for thin sectioning. Thin sections were cut with a glass knife on a LKB Ultratome III, poststained with uranyl acetate and lead citrate followed by examination with a Hitachi HU-11E electron microscope at a working voltage of 75KV.

Statistical Analyses

Data were analyzed by least squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (Barr et al., 1979). The overall mathematical model included effects of Status (pregnant vs. nonpregnant), Day and, Status by Day interactions. Data were then separated by Status. Results for nonpregnant flushings were analyzed for Day trends, whereas data from pregnant uterine flushings were evaluated relative to stages in blastocyst development since changes in uterine flushings were associated more closely with blastocyst size than actual day of pregnancy.

RESULTS

General

Data from uterine flushings of both pregnant and nonpregnant pigs were compared initially relative to Day post-estrus (10.5, 11.0, 11.5, 12.0 and 14.0). An overall analysis of variance indicated that Status x Day interactions only approached significance. However, Bartlett's test for homogeneity of variance indicated that variances were heterogeneous ($P<0.01$) due to greater variation within the pregnant group. The large amount of variation in stage of blastocyst development (spherical, tubular and filamentous) with Day contributed to greater variation in the various responses, e.g., E_2, associated with Day. Therefore, data from pregnant gilts were analyzed relative to average blastocyst size within a uterine horn at the time of collection of the uterine flushing: <5 mm spherical; 5–8 mm spherical 9–50 mm tubular; >50 mm Day 12 filamentous and Day 14 filamentous. Uterine flushings from nonpregnant gilts were analyzed relative to days of the estrous cycle which were equivalent to the respective stages of blastocyst development, i.e., Days 10 to 14. Statistical analyses indicated that partitioning of responses based upon blastocyst size for pregnant gilts resulted in detection of significant Status x Day interactions.

Estrogens

Data on total recoverable free and conjugated estrogens in uterine flushings from pregnant and nonpregnant gilts are summarized in Table 1.

Day trends for total recoverable E_1, E_2 and E_3 in nonpregnant uterine flushings were not significant ($P>0.10$). However, total recoverable E_2 in pregnant uterine flushings was significantly ($P<0.01$) affected by stage of blastocyst development. Total E_2 ($\bar{X}$) increased almost 4-fold in flushings with tubular blastocysts (2.3 ng) as compared to flushings with spherical blastocysts (0.6 ng), continued to increase in uterine flushings with filamentous blastocysts (4.4 ng) on Day 12 and then declined by Day 14 (0.4 ng). Though nonsignificant statistically, changes in total E_1 and E_3 followed a similar pattern, but an obvious increase was not detected until uterine flushings contained filamentous blastocysts (Day 12). Detectable E_3 was not always present in pregnant uterine flushings; however, the presence of E_3 was associated with blastocyst elongation. Only 2 of 15 uterine flushings with spherical blastocysts had detectable E_3, while 5 of 8 with tubular blastocysts and 6 of 9 with filamentous blastocysts had E_3. Estriol was detected in only 2 of 25 uterine flushings from nonpregnant gilts.

Total recoverable conjugated estrogens in nonpregnant flushings were not significantly affected by Day of the estrous cycle, although E_1S appeared to be higher on Day 14. Total E_1S and E_2S in pregnant uterine flushings were affected significantly ($P<0.01$) by stage of blastocyst development. Total E_1S and E_2S were higher in uterine flushings containing tubular and filamentous blastocysts; however, values declined on Day 14. Although E_3S appeared similar among spherical, tubular and Day 12 filamentous blastocysts, the percentage of uterine flushings with detectable E_3S increased as blastocyst development progressed (Table 1).

Protein, Acid Phosphatase, Ca++, PGF and PGE_2

Data relative to total recoverable calcium, protein, acid phosphatase, PFG and PGE_2 in pregnant and nonpregnant uterine flushings are summarized in Table 2.

Significant Day trends were detected for total acid phosphatase ($P<0.01$), protein ($P<0.01$) and PGE_2 ($P<0.01$) in uterine flush-

TABLE 1. Changes in estrogens in uterine flushings from pregnant and nonpregnant gilts ($\bar{X}$ ± SEM).

	Nonpregnant (Day)[a]					Pregnant (blastocyst size, mm/Day)[abc]				
						5.0	5.0– 8.0	Tubular	Filamentous	
Item (ng)	10.5	11.0	11.5	12.0	14.0	10.5	10.5–11.0	11.0–12.0	11.5–12.0	14
Total E_1	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.04	0.4 ± 0.01	0.2 ± 0.03	0.5 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	2.8 ± 1.0	0.6 ± 0.10
Total E_2	0.4 ± 0.1	0.5 ± 0.3	0.2 ± 0.04	0.4 ± 0.2	0.2 ± 0.1	0.7 ± 0.3	0.6 ± 0.1	2.3 ± 0.5	4.4 ± 1.6	0.4 ± 0.10
Total E_3	1.5	1.4	ND[d]	ND	ND	3.2	2.0	1.1 ± 0.5	5.5 ± 0.1	ND
	(1/9)[e]	(1/3)	(0/3)	(0/7)	(0/3)	(1/8)	(1/7)	(5/8)	(6/9)	(0/2)
Total E_1S	0.9 ± 0.3	0.9 ± 0.5	0.6 ± 0.1	0.5 ± 0.1	1.6 ± 0.8	0.8 ± 0.2	1.3 ± 0.4	3.4 ± 0.8	2.2 ± 0.3	0.8 ± 0.2
Total E_2S	1.0 ± 0.4	0.3 ± 0.04	0.5 ± 0.2	0.3 ± 0.1	0.1 ± 0.01	0.5 ± 0.2	0.5 ± 0.2	1.7 ± 0.4	1.6 ± 0.3	0.2 ± 0.1
Total E_3S	0.6 ± 0.2	ND	1.1	ND	ND	1.8 ± 0.6	1.4 ± 0.2	1.6 ± 0.3	1.5 ± 0.5	ND
	(3/9)	(0/3)	(1/3)	(0/7)	(0/3)	(2/8)	(2/7)	(6/8)	(5/9)	(0/2)

[a]Significant ($P<0.05$) Day × Status interaction for E_2, E_1S and E_2S.

[b]For pregnant gilts trends related to blastocyst size significant ($P<0.01$) for E_2, E_1S and E_2S.

[c]An approximate relationship between day of gestation and blastocyst size.

[d]ND=not detected.

[e]Number of gilts with detectable E_3 and E_3S in uterine flushings.

TABLE 2. Changes in calcium, protein, acid phosphatase and prostaglandins E_2 and F of uterine flushings from pregnant and nonpregnant gilts ($\bar{X}$ ± SEM).

	Nonpregnant (Day)[ab]					Pregnant (blastocyst size, mm/Day)[acd]				
						<5.0	5–8	Tubular	Filamentous	
Item	10.5	11.0	11.5	12.0	14.0	10.5	10.5–11.0	11.0–12.0	12	14
Total Calcium (mg)	0.2 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.1 ± 0.1	0.6 ± 0.2	1.5 ± 0.2	0.8 ± 0.2	0.1 ± 0.1
Total Protein (mg)	13 ± 3	8 ± 2	16 ± 6	13 ± 2	55 ± 10	13 ± 3	11 ± 2	31 ± 9	35 ± 4	69 ± 12
Total Acid Phosphatase[e]	3 ± 1	9 ± 3	5 ± 2	9 ± 5	34 ± 5	3 ± 1	4 ± 1	23 ± 7	17 ± 3	16 ± 3
Total PGF (ng)	2 ± 0.2	2 ± 0.2	3 ± 1	3 ± 1	4 ± 2	1 ± 0.4	3 ± 0.3	6 ± 1	27 ± 13	108 ± 23
Total PGE_2 (ng)	1 ± 0.4	1 ± 0.3	1 ± 0.3	2 ± 0.4	3 ± 0.2	3 ± 1	16 ± 6	24 ± 6	66 ± 19	197 ± 12

[a]Significant day × status interaction for Ca, PGF and PGE_2 ($P<0.01$), Pr ($P<0.10$) and Day trends for AP ($P<0.05$).

[b]For nonpregnant gilts day trends were significant for Pr ($P<0.01$) and PGE_2 ($P<0.05$).

[c]For pregnant gilts trends related to blastocyst size were significant ($P<0.01$) for Pr, Ca, PGF, PGE_2.

[d]An approximate relationship between day of gestation and blastocyst size.

[e]μ mole Pi released/min using *p*-nitrophenyl phosphate as substrate at 30°C.

ings from nonpregnant gilts. Acid phosphatase activity in nonpregnant uterine flushings increased between Days 10.5 and 14. Protein and PGE_2 contents were similar between Days 10.5 and 12 and then increased on Day 14.

Stage of blastocyst development had a significant ($P<0.01$) effect on total recoverable calcium, protein, acid phosphatase, PGF and PGE_2. Total calcium increased in flushings with tubular and Day 12 filamentous blastocysts and then declined by Day 14. Total protein, PGF and PGE_2 increased in a similar pattern between Days 10.5 and 12, and continued to increase through Day 14 of pregnancy. Although a significant Day trend ($P<0.05$) for total PGE_2 was detected in nonpregnant and pregnant uterine flushings, total PGE_2 was almost 12 times higher in pregnant flushings containing Day 12 filamentous blastocysts as compared with Day 12 nonpregnant uterine flushings, and this difference was almost 60 times greater for pregnant compared with nonpregnant uterine flushings by Day 14.

Qualitative Analyses of Uterine Protein

Basha et al. (1980) reported results of mapping acidic and basic polypeptides found in porcine uterine flushings following 2D-PAGE. Comparisons of Coomassie blue-stained acidic proteins (IEF) suggest that pregnant and nonpregnant uterine flushings from Days 12 and 14 contain the same major protein components (Fig. 1). The dominant protein seen on all gels is albumin, with several serum-like polypeptides also present. Two proteins of 15,000 to 17,000 M_r and pI's of 6.1 and 6.3 were detected in pregnant and nonpregnant uterine flushings, but not serum. Basha et al. (1980) indicated that these two proteins were

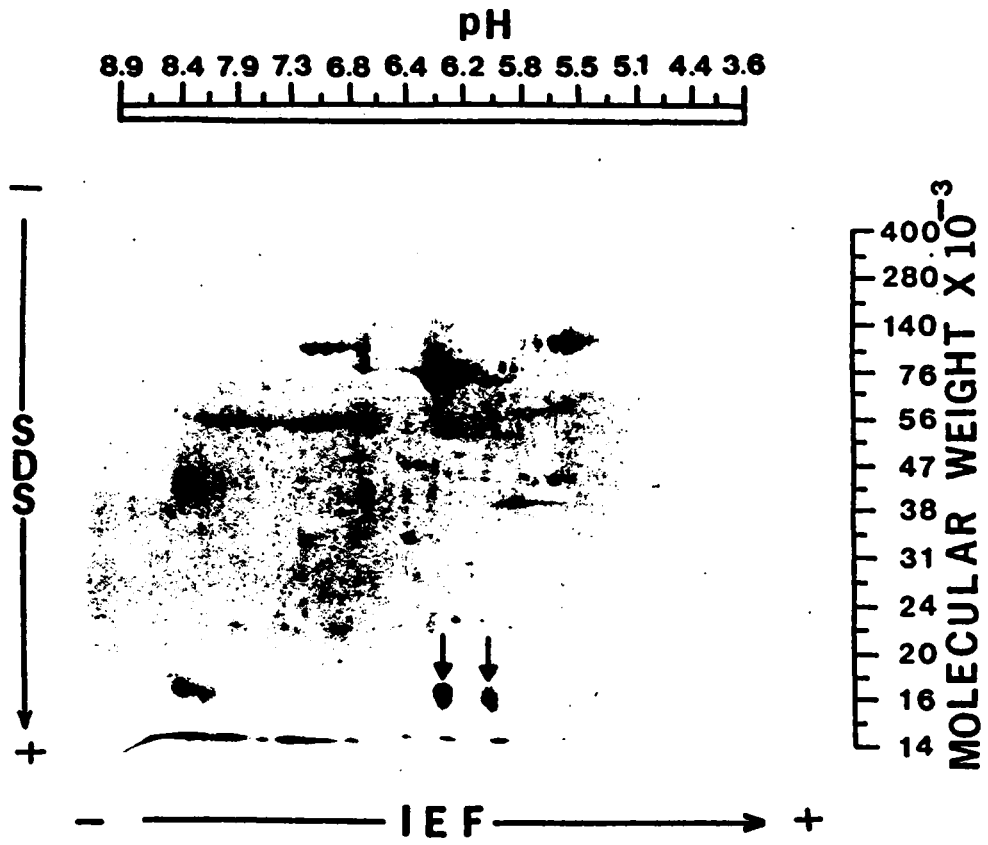

FIG. 1. Two-dimensional gel electrophoresis of acid proteins *(IEF)* characteristic of uterine flushings from Day 12 of pregnancy. Two nonserum proteins with molecular weights of about 15,000 to 17,000 are present *(arrows)* with serum albumin *(A)* the major protein. An identical protein profile was obtained when uterine flushings from Day 12 of the estrous cycle were analyzed by IEF when 300 μg total protein was used.

progesterone-induced; however, their function is not known.

Basic proteins *(NEPHGE)* found in pregnant and nonpregnant uterine flushings are presented in Fig. 2. The appearance of uteroferrin ($M_r \cong$ 32K, designated *U*) and two other nonserum proteins ($M_r \cong$ 50K to 60K pI'S $\cong$ 7.8, designated as proteins 1 and 2) were closely associated with initiation of estrogen secretion by tubular and Day 12 filamentous blastocysts. In nonpregnant gilts, these proteins were detected in only 1 of 6 uterine flushings on Day 12 and in all Day 14 uterine flushings. These proteins were also present in Day 14 pregnant uterine flushings, but the staining intensity was less than on Day 12. These observations indicate the clear influence of blastocyst estrogens on initiation of endometrial protein secretion.

Ultrastructural Changes in Endometrium

Onset of blastocyst elongation in pregnant gilts (tubular stage) was associated with ultrastructural changes in the surface and glandular endometrial epithelium. The luminal surface epithelium from Day 12 nonpregnant endometrium and/or endometrium associated with spherical blastocysts appeared to have a rounded surface and an abundance of microvilli with various amounts of what appeared to be holocrine secretion and protrusions on the surface (Fig. 3A). Surface epithelium of pregnant gilts associated with tubular and filamentous blastocysts appeared flattened and hexagonal in shape with a reduction in diameter of the microvilli (Fig. 3B). Cell debris was lacking on the smooth epithelial surface and stereocilia were abundant.

Uterine glandular epithelium of endometrium with spherical blastocysts had an accumulation of clear vesicles in the supranuclear region (Fig. 4A). Electron-dense vesicles which may contain lipid were present below the nucleus. The lumina of the glands at this stage are very small and generally free of cellular material. Elongation of blastocysts was associated with release of the clear secretory vesicles (Fig. 4B), widening of the glandular lumen and the appearance of large amounts of cellular debris. The clear supranuclear vesicles were less abundant in the glandular epithelium at more advanced stages of blastocyst elongation (Day 12 filamentous), but both the Golgi apparatus and rough endoplasmic reticulum were still prominent, suggesting that active synthesis of proteins was still occurring (Fig. 4C). Glandular epithelium of Day 12 nonpregnant endometrium continued to have an accumulation of clear vesicles in the supranuclear region (Fig. 4D) and there was no indication of a synchronized release of cellular secretory material comparable to that exhibited by pregnant endometrial glandular epithelium when tubular blastocysts were present.

DISCUSSION

Results of the present study indicated that the initial increase in estrogen content of uterine flushings occurred when blastocysts reached approximately 10 mm in diameter, i.e., the late spherical stage at about Day 11.5 of pregnancy. This is consistent with earlier results (Zavy et al., 1980) indicating an increase in estrogens in uterine flushings between Days 10 and 12 of gestation in gilts. The time of onset of estrogen production by pig blastocysts coincides with the time of maternal recognition of pregnancy (Dhindsa and Dziuk, 1968; Bazer and Thatcher, 1977; Flint et al., 1979) and just precedes initiation of blastocyst elongation.

Initial in vitro production of estrone and estradiol (Flint et al., 1979) from conversion of androstenedione to these steroids occurred when blastocysts were greater than 5 mm in diameter. Our in vivo results indicate that estradiol content of uterine flushings increased ($P<0.01$) when blastocysts were 9–10 mm in diameter as compared with earlier stages in development. Fischer (1981) indicated conversion of progesterone to estrogens by spherical blastocysts measuring 9–10 mm as compared with little or no detectable estrogen production by blastocysts that measured 5–7 mm. Fischer (1981) also found estrogen production by Day 12 filamentous blastocysts, but conversion of progesterone to estrogens appeared to be less than that for tubular blastocysts. The apparent decrease in estrogen synthesis noted by Fischer (1981) is consistent with the decrease in total recoverable estrogens in uterine flushings on Day 14 in the present study.

Changes in uterine luminal content of conjugated estrogens have not been previously reported. Total recoverable conjugated estrogens in nonpregnant flushings did not change significantly between Days 10 and 12 of the estrous cycle. However, estrone sulfate was elevated on Day 14 which may reflect the rise in follicular estrogen production from the ovary (Hansel et

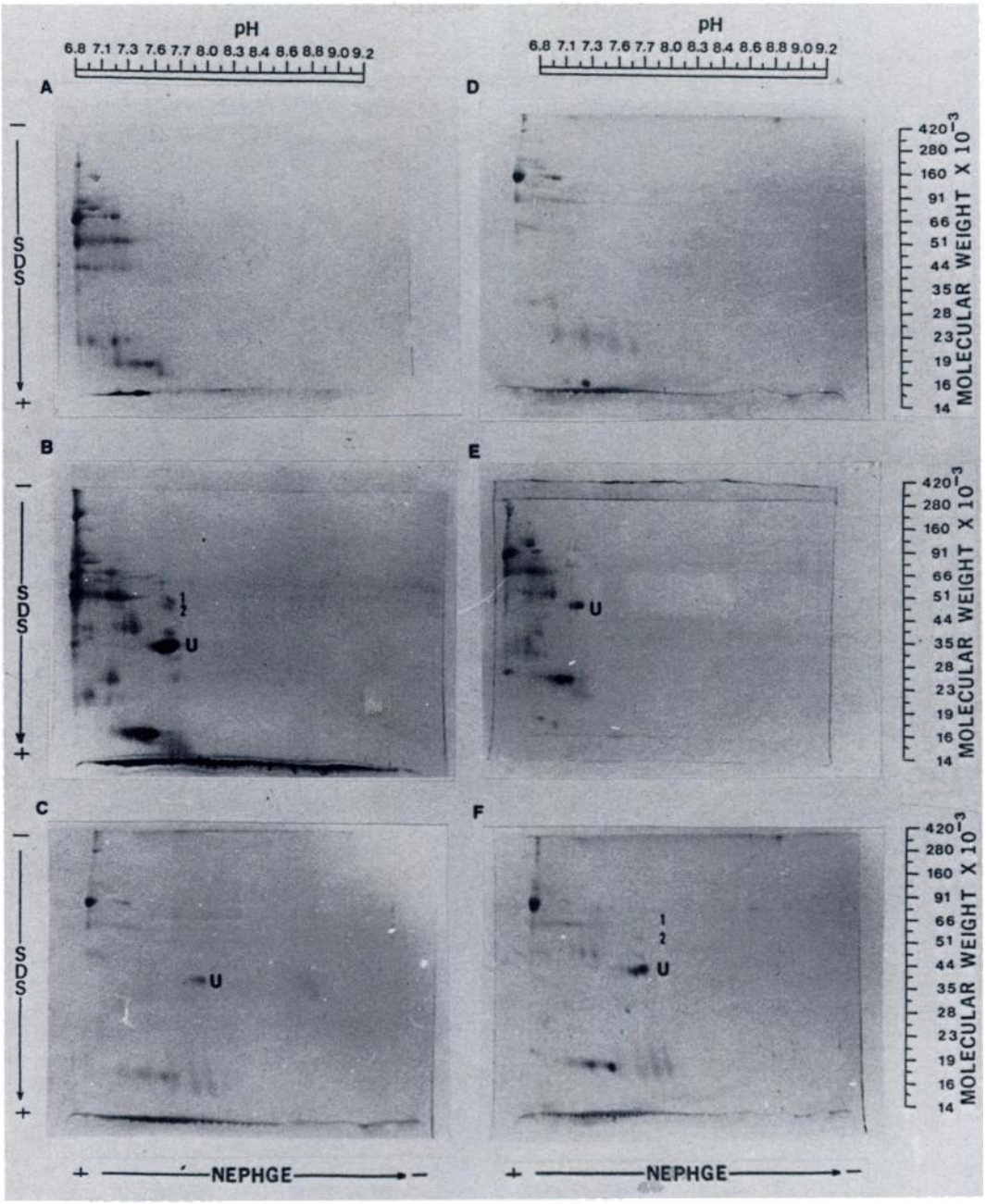

FIG. 2. Two-dimensional polyacrylamide gel electrophoresis of basic proteins *(NEPHGE)* in uterine flushings from pregnant and nonpregnant gilts. A total of 300 μg total protein was used for each NEPHGE gel. Uteroferrin *(U)* and basic proteins *1* and *2* were compared since effects of sex steroids on their appearance has been established (Basah et al., 1980). Plates *A*, *B* and *C* are gels of flushings which contained spherical *(A)*, tubular or Day 12 filamentous blastocysts *(B)* and Day 14 filamentous blastocysts *(C)*. Plates *D*, *E* and *F* represent gels of flushings obtained from nonpregnant gilts on Days 10 *(D)*, 12 *(E)* and 14 *(F)* of the estrous cycle. Uteroferrin and basic proteins 1 and 2 were observed in uterine flushings from pregnant gilts which contained tubular blastocysts *(B)* and in Day 14 nonpregnant uterine flushings *(F)*. Although these gels do not allow for quantitative comparisons, apparent differences in staining intensity are due in part to changes in the relative contribution of basic proteins to total protein in uterine flushings from nonpregnant and pregnant gilts (see discussion and Knight et al., 1973; Basha et al., 1979; Zavy et al., 1980).

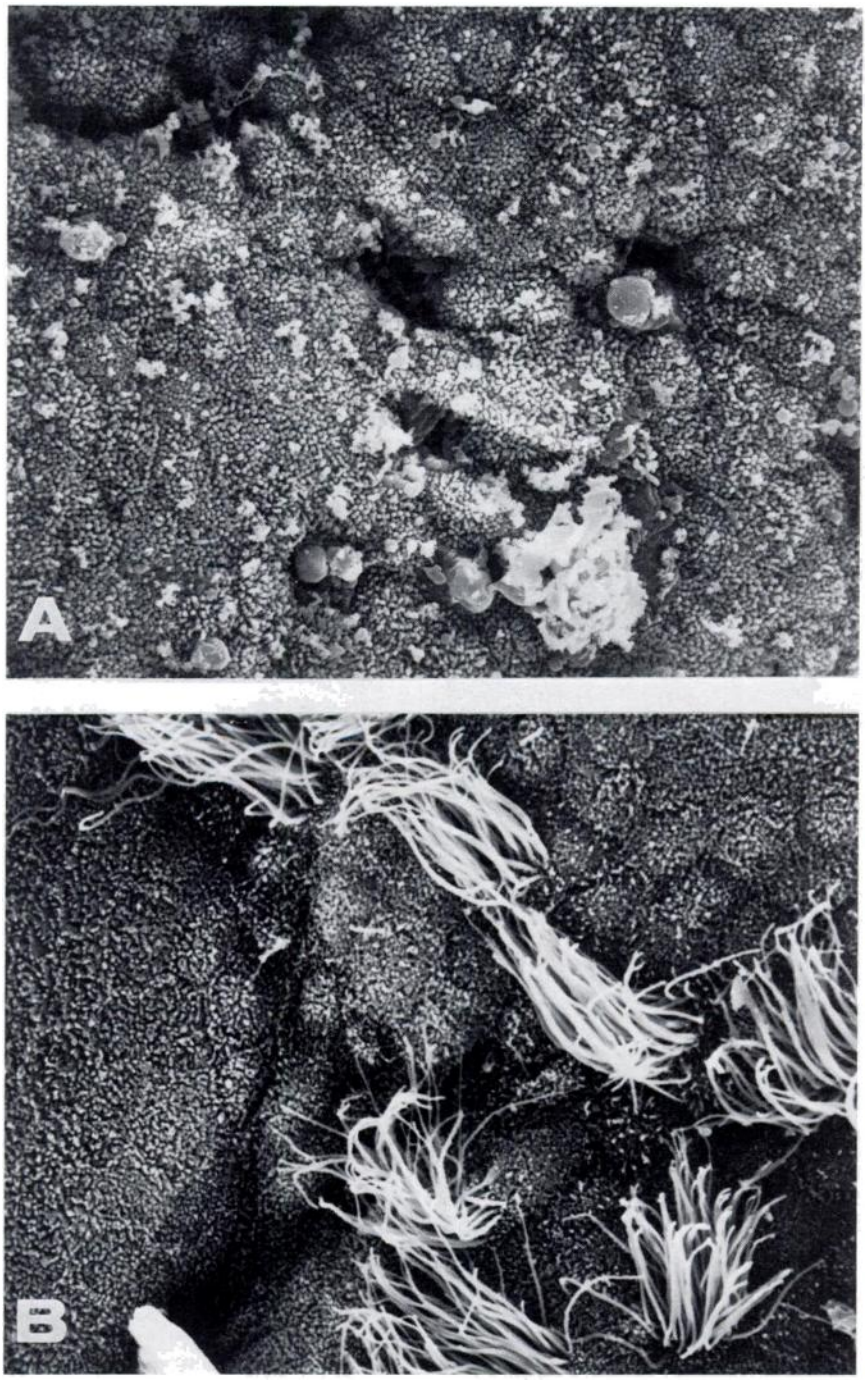

FIG. 3. Scanning electron micrographs of uterine endometrium. *A)* Uterine luminal surface epithelium from a gilt on Day 12 of the estrous cycle. Uterine luminal epithelium from pregnant gilts with spherical blastocysts had very similar morphological characteristics. *B)* Luminal surface epithelium from a Day 12 pregnant gilt from which tubular and filamentous blastocysts were recovered in uterine flushings. Note the flattened and hexagonal shape of the epithelial cells. × 3100.

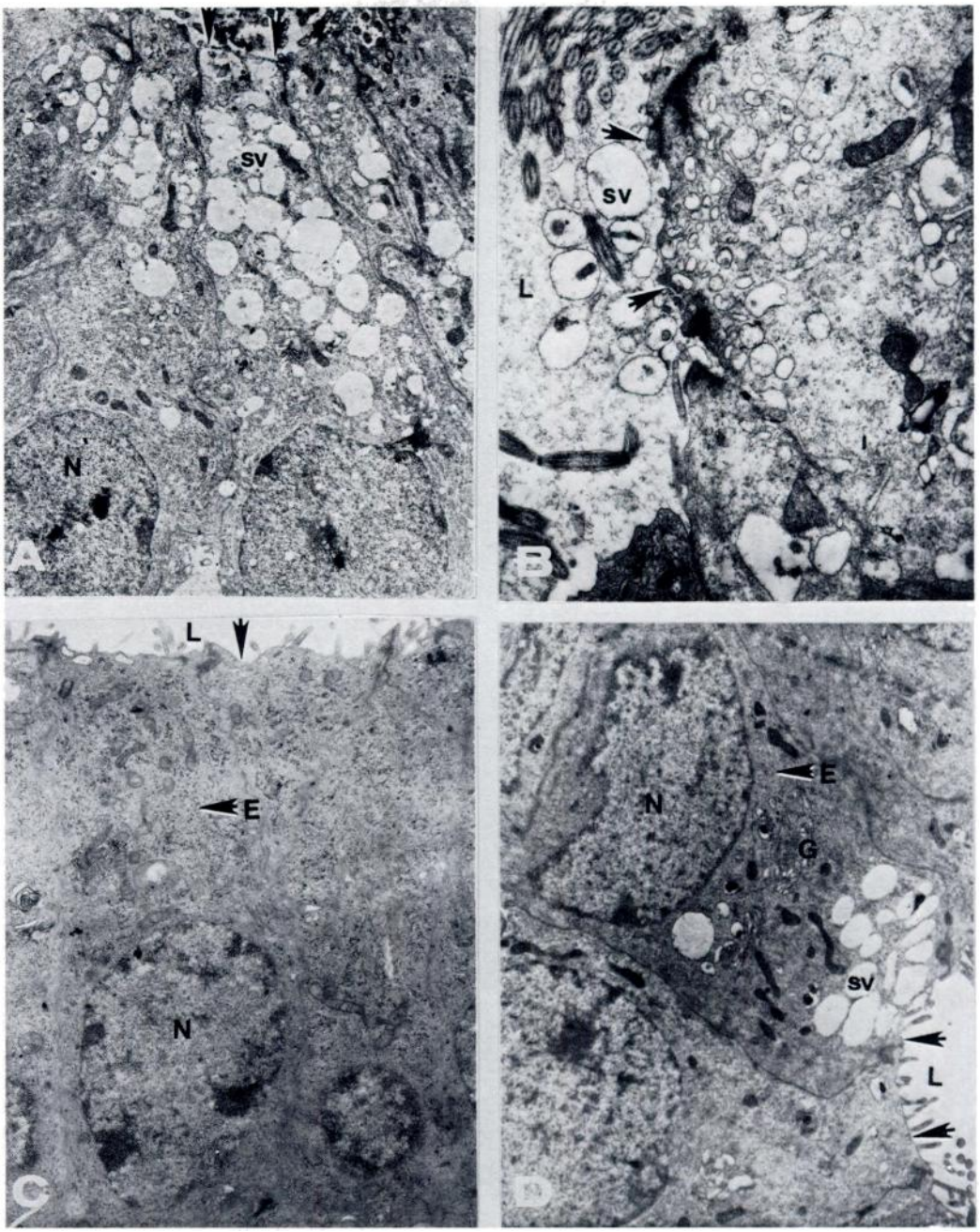

FIG. 4. Transmission electron micrographs of uterine glandular epithelium. *A)* Glandular epithelial cells of a Day 11 pregnant uterus which contained spherical blastocysts. X 7800. Note the numerous intracellular secretory vesicles *(sv)* in the supranuclear portion of the cell. The lumen *(L)* of the uterine gland contains cross-sections of stereocilia and the epithelial cell membranes surface adjacent to the lumen is noted by arrows. *B)* Exocytosis of secretory vesicles *(sv)* into the lumen *(L)* of the uterine gland from epithelial cells from a uterus which contained tubular blastocysts. The epithelial cell membrane is noted by the *arrows* and cross-sections of stereocilia are present in the lumen. X 20,000. *C)* Uterine glandular epithelial cells after blastocyst elongation on Day 12. Secretory vesicles are absent, but the endoplasmic reticulum *(arrow E)* is well developed. The gland lumen *(L)* and cell nucleus *(N)* are also noted. X 14,000. *D)* Uterine gland epithelial cells from a Day 12 non-pregnant gilt. Numerous intracellular secretory vesicles *(sv)* are located in the supranuclear region of the cell and near the cell membrane *(arrows)*. X 7800. Other cellular structures are the nucleus *(N)*, endoplasmic reticulum *(arrow E)*, golgi *(G)* and gland lumen *(L)*.

al., 1973). The uterine endometrium contains 17β-hydroxysteroid dehydrogenase and sulphotransferase activity (Heap and Perry, 1974; Pack and Brooks, 1974) which could convert free estrogens produced by the ovary to estrone sulfate as they move through the uterine endometrium. Conjugated estrogen content in pregnant uterine flushings increased concurrently with the increase in free estrogen content which would reflect endometrial sulfation of the blastocyst "free" estrogens. This concept would suggest that elevated plasma E_1S concentrations should be detectable between Days 11 and 12 of pregnancy. Detectable increases in unconjugated and conjugated estrogens in the peripheral circulation of pregnant animals occur by Day 16 (Robertson and King, 1974; Robertson et al., 1978) which is well after initiation of blastocyst estrogen production on Day 12 (Heap et al., 1981; Zavy et al., 1980). However, Stoner et al. (1981) reported a significant increase in E_1S concentrations in utero-ovarian vein plasma between Days 11 and 12 of pregnancy followed by a decline until Day 16. The increase in utero-ovarian vein plasma E_1S concentrations between Days 11 and 12 of pregnancy are consistent with increased luminal content of conjugated and unconjugated estrogens detected in the present study. Conversion of blastocyst estrogens by conjugation to a biologically inactive form before entering the maternal circulation would suggest a local effect of blastocyst estrogens on the uterine endometrium. The increase in uterine luminal content of calcium and protein in the present study also indicated that a local response occurs. Estrogens have been shown to influence the movement of calcium in cells of rat endometrium (Pietras and Szego, 1975) and calcium is known to influence the release of secretory vesicles from cells of the adrenal medula and cortex, salivary gland, pancreas, kidney and nerve cells (see reviews by Rasmussen and Goodman, 1977; and Rubin and Laychock, 1978).

Rubin and Laychock (1978) proposed that calcium, released from the plasmalemma of adrenal cortex when surface receptors are activated, is involved directly with activation of phospholipase A_2 to enhance prostaglandin synthesis and indirectly to induce exocytosis of secretory vesicles. These authors also indicated an increase in both $PGF_2\alpha$ and PGE_2 during ACTH stimulation of the adrenal cortex. The pattern of change in calcium, protein, PGF and PGE_2 content in uterine flushings of pregnant gilts in the present study suggests that blastocyst estrogens may function in a similar manner to induce endometrial secretion of histotroph into the uterine lumen to provide a nutrient source for developing conceptuses.

Steroid stimulated release of secretory material from the uterine glandular epithelium has been reported previously in the cat (Bareither and Verhage, 1980). These authors indicated that progesterone stimulated the release of secretory material from cat uterine epithelium synthesized under the influence of estradiol. Results of the present study indicate that estrogen from the blastocysts may stimulate release of endometrial secretory proteins in a manner similar to that induced by progesterone in the cat. Although the model of ACTH activation of secretion involves surface receptors, estrogen could bring about release of calcium through perturbation of the membrane (Pietras and Szego, 1977). However, Pietras and Szego (1977) indicated that binding sites for estrogen are present on the cell membrane of rat uterine epithelium.

The increase in total PGF in pregnant uterine flushings supports the findings of Zavy et al. (1980). This increase in uterine luminal content of PGF is associated with increased estrogen production by blastocysts in pregnant gilts (Zavy et al., 1980) or estrogen injections in nonpregnant gilts (Frank et al., 1977, 1978) which may allow for maintenance of corpora lutea (Bazer and Thatcher, 1977).

Total PGE_2 in pregnant uterine flushings increased during blastocyst elongation and occurred simultaneously with blastocyst estrogen production. This increase in PGE_2 and PGF content may reflect either secretion of PGE_2 and PGF from uterine epithelium and/or blastocyst (Watson and Patek, 1979) into the pregnant uterine lumen. Nkuuhe and Manns (1981) demonstrated that PGE_1 influences the flux of fluid and $PGF_2\alpha$ across the uterine epithelium in the ewe. Prostaglandin E_1 stimulated fluid movement into the uterine lumen and reduced $PGF_2\alpha$ flux from the uterine lumen and into the uterine vasculature of ewes.

The presence of three basic nonserum proteins was associated with increased estrogen content of uterine flushings containing tubular and Day 12 filamentous blastocysts. The major basic protein present was uteroferrin (Buhi et al., 1979) which is a progesterone-induced glycoprotein (Knight et al., 1973). Characteri-

zation and function of this glycoprotein in iron transport have been described previously (Schlosnagle et al., 1974; Roberts and Bazer, 1980). The appearance of uteroferrin and two other basic polypeptides detected on 2D-PAGE gels was associated with initiation of blastocyst estrogen synthesis in pregnant pigs. These polypeptides were detected in all flushings when tubular and Day 12 filamentous blastocysts were present, but were not seen in flushings with spherical blastocysts or until Day 14 of the estrous cycle. Appearance of these proteins, therefore, seems to be influenced by increasing estrogen levels which is consistent with previous observations (Basha et al., 1980). Basha et al. (1980) indicated that uteroferrin was present in uterine secretions of ovariectomized gilts treated with progesterone for 15 days, but the other two basic proteins (M_r 50K) were absent. However, these proteins were detected in intact gilts which were treated with estradiol valerate to induce prolonged maintenance of corpora lutea.

The appearance of these proteins on Day 14 of the estrous cycle occurs when peripheral ovarian estrogens are increasing (Hansel et al., 1973; Zavy et al., 1980). The decrease in uteroferrin and the other two basic polypeptides on Day 14 of pregnancy in the present study is consistent with the observation that estrogen suppresses synthesis and/or secretion of uteroferrin (Knight et al., 1973; Basha et al., 1979). This depression of uteroferrin is particularly evident based on decreased staining intensity on 2D-PAGE gels (Fig. 2) and reduced acid phosphatase activity in uterine flushings on Day 14 of pregnancy (Table 2). Measurement of acid phosphatase activity reflects uteroferrin content since 95% of this enzyme activity in the uterus is contributed by uteroferrin (Basha et al., 1979). It would appear, therefore, that the synthesis of blastocyst estrogens initially regulates the release of specific uterine proteins at Day 12 of pregnancy followed later by modification of their synthesis and/or secretion.

An estrogen-stimulated release of proteins during blastocyst elongation coincides with ultrastructural changes. The synchronized release of material from the glandular epithelium parallels increases in intraluminal estrogens and calcium. Histochemical identification of material in these vesicles has not been reported. Secretion of protein also is occurring in nonpregnant gilts; however, there was no evidence for synchronized release of secretory material. The flattening of the surface epithelium observed by SEM could contribute to the increased endometrial surface area and length of uterine horns between Days 12 and 18 of pregnancy (Perry and Rowlands, 1962).

Biochemical and histological changes during pig blastocyst elongation bear a strking resemblance to those associated with termination of delayed implantation in roe deer (see Aitken, 1977, for review). During activation of blastocyst development in the roe deer, estrogen, calcium and protein content become elevated in uterine flushings and ultrastructural studies indicated release of secretory vesicles from the glandular epithelium. Gadsby et al. (1980) reported that the allantochorionic placenta of the roe deer possesses aromatase activity and has the ability to produce estrogens. The present study indicates the possibility that a common mechanism for blastocyst activation of endometrial function may exist between the two species although pig blastocysts do not enter a stage of delayed development.

In summary, results of this study indicate that initial elongation of pig blastocysts is associated with increased estrogen content in the uterine lumen. Blastocyst estrogens may stimulate release of free calcium from the uterine epithelium allowing for increased prostaglandin production and a synchronized release of secretory proteins into the uterine lumen. The local effect of blastocyst stimulation would provide for continued production of endometrial secretions needed by the conceptus for nourishment and allow for maintenance of corpora lutea and the establishment of pregnancy.

REFERENCES

Aitken, R. J. (1977). Embryonic diapause. In: Development in Mammals, vol. 1 (M. H. Johnson, ed.). North-Holland, Amsterdam, pp. 307–360.

Alexander, R. L. (1971). Evaluation of an automatic calcium titrator. Clin. Chem. 17:1171–1175.

Anderson, L. L. (1978). Growth, protein content and distribution of early pig embryos. Anat. Rec. 190:143–154.

Bareither, M. L. and Verhage, H. G. (1980). Effect of estrogen and progesterone on secretory granule formation and release in the endometrium of the ovariectomized cat. Biol. Reprod. 22:635–643.

Barr, A. J., Goodnight, J. H., Sall, J. P., Blair, W. H. and Chilko, D. M. (1979). SAS User's Guide. SAS Institute Inc., Raleigh, NC.

Basha, S.M.M., Bazer, F. W. and Roberts, R. M. (1979). The secretion of a uterine specific, purple phosphatase by cultured explants of porcine endometrium. Dependency upon the state of

pregnancy of the donor animal. Biol. Reprod. 20:431–441.

Basha, S.M.M., Bazer, F. W., Geisert, R. D. and Roberts, R. M. (1980). Progesterone-induced uterine secretions in pigs. Recovery from pseudo-pregnant and unilaterally pregnant gilts. J. Anim. Sci. 50:113–123.

Bazer, F. W. and Thatcher, W. W. (1977). Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin $F_2\alpha$ by the uterine endometrium. Prostaglandins 14:397–401.

Buhi, W., Bazer, F. W., Ducsay, C. D., Chun, P. W. and Roberts, R. M. (1979). Iron content, molecular weight and possible function of progesterone-induced purple glycoprotein of the porcine uterus. Fed. Proc. 38:733.

Chenault, J. R., Thatcher, W. W., Kalra, P. S., Abrams, R. M. and Wilcox, C. J. (1975). Transitory changes in plasma progestins, estradiol, and luteinizing hormone approaching ovulation in the bovine. J. Dairy Sci. 58:709–717.

Cornette, J. C., Kirton, K. T., Barr, K. L. and Forbes, A. D. (1972). Radioimmunoassay of prostaglandins. J. Reprod. Med. 9:355–360.

Crombie, P. R. (1972). The morphology and ultrastructure of the pig's placenta throughout pregnancy. Ph.D. Dissertation, University of Cambridge, Cambridge, England.

Dhindsa, D. W. and Dziuk, P. J. (1968). Effect on pregnancy in the pig after killing embryos or fetuses in one uterine horn in early gestation. J. Anim. Sci. 27:122–126.

Eley, D. S., Thatcher, W. W., Head, H. H., Collier, R. J. and Wilcox, C. J. (1981). Periparturient endocrine changes of conceptus and maternal units in Jersey cows bred for milk yield. J. Dairy Sci. 64:296–311.

Eley, R. M., Thatcher, W. W. and Bazer, F. W. (1979). Hormonal and physical changes associated with bovine conceptus development. J. Reprod. Fertil. 55:191–190.

Fischer, H. E. (1981). Metabolism of progesterone to estrogens in porcine uterine endometrial and conceptus tissues. M.S. Thesis, University of Florida, Gainesville, FL.

Flint, A.P.F., Burton, R. D., Gadsby, J. E., Saunders, P.T.K. and Heap, R. B. (1979). Blastocyst oestrogen synthesis and the maternal recognition of pregnancy. In: Maternal Recognition of Pregnancy, Ciba Foundation Symposium 64 (J. Whelan, ed.) Excerpta Medica, Amsterdam, pp. 209–238.

Frank, M., Bazer, F. W., Thatcher, W. W. and Wilcox, C. J. (1977). A study of prostaglandin $F_2\alpha$ as the lyteolysin in swine: III. Effects of estradiol valerate on prostaglandin F, progestins, estrone, and estradiol concentrations in the utero-ovarian vein of nonpregnant gilts. Prostaglandins 14: 1183–1196.

Frank, M., Bazer, F. W., Thatcher, W. W. and Wilcox, C. J. (1978). A study of prostaglandin $F_2\alpha$ as the luteolysin in swine: IV. An explanation for the luteotrophic effect of estradiol. Prostaglandins 15:151–159.

Gadsby, J. E., Heap, R. B. and Burton, R. D. (1980). Oestrogen production by blastocyst and early embryonic tissue of various species. J. Reprod. Fertil. 60:409–417.

Guthrie, H. D., Henricks, D. M. and Handlin, D. L. (1972). Plasma estrogen, progesterone and luteinizing hormone prior to estrus and during early pregnancy in pigs. Endocrinology 91:675–679.

Hansel, W., Concannon, P. W. and Lukaszewska, J. H. (1973). Corpora lutea of the large domestic animals. Biol. Reprod. 8:222–245.

Heap, R. B. and Perry, J. S. (1974). The maternal recognition of pregnancy. Br. J. Hosp. Med. 12:8–12.

Heap, R. B., Flint, A.P.E., Hartmann, P. E., Gadsby, J. E., Staples, L. D., Ackland, N. and Hamon, M. (1981). Oestrogen production in early pregnancy. J. Endocrinol. 89:77–94.

Horst, M. N. and Roberts, R. M. (1979). Solubilization, electrofocusing and two-dimensional electrophoresis of plasma membrane polypeptides from Chinese hamster ovary cells. In: Electrofocus/78 (B. Haglund, H. Westerfeld and J. Ball, Sr., eds.). Elsevier/North Holland, Amsterdam, pp. 99–117.

Horst, M. N., Basha, S.M.M., Baumbach, G. A., Mansfield, E. H. and Roberts, R. M. (1980). Alkaline urea solubilization, two-dimensional electrophoresis and lectin staining of mammalian cell plasma membrane and plant seed proteins. Anal. Biochem. 102:399–408.

Knight, J. W., Bazer, F. W. and Wallace, H. D. (1973). Hormonal regulation of porcine uterine protein secretions. J. Anim. Sci. 36:546–553.

Knight, J. W., Bazer, F. W., Thatcher, W. W., Franke, D. E. and Wallace, H. D. (1977). Conceptus development in intact and unilaterally hysterectomized-ovariectomized gilts: interrelations among hormonal status, placental development, fetal fluids and fetal growth. J. Anim. Sci. 44:620–637.

Lewis, G. S., Jenkins, P. E., Fogwell, R. L. and Inskeep, E. K. (1978). Concentrations of prostaglandins E_2 and $F_2\alpha$ and their relationship to luteal function in early pregnant ewes. J. Anim. Sci. 47:1314–1323.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with folin phenol reagent. J. Biol. Chem. 193:265–275.

Moeljono, M.P.E., Thatcher, W. W., Bazer, F. W., Frank, M., Owens, L. J. and Wilcox, C. J. (1977). A study of prostaglandin $F_2\alpha$ as the luteolysin in swine: II. Characterization and comparison of prostaglandin F, estrogen and progestin concentrations in uteroovarian vein plasma of nonpregnant gilts. Prostaglandins 14:543–555.

Murray, F. A., Bazer, F. W., Wallace, H. D. and Warnick, A. C. (1972). Quantitative and qualitative variation in the secretion of protein by the porcine uterus during the estrous cycle. Biol. Reprod. 7:314–320.

Nkuuhe, J. and Manns, J. (1981). Effect of prostaglandin (PGE_1) on fluid and $PGF_2\alpha$ fluxes across the uterine epithelium in the ewe. Proceedings 14th Annual Meeting Society for the Study of Reproduction, Corvallis, OR, Abstr. #263.

O'Farrell, P. H. (1975). High resolution two-dimen-

sional electrophoresis of proteins. J. Biol. Chem. 250:4007–4021.

Pack, B. A. and Brooks, S. C. (1974). Cyclic activity of estrogen sulfotransferase in the gilt uterus. Endocrinology 95:1680–1690.

Perry, J. S. and Rowlands, I. W. (1962). Early pregnancy in the pig. J. Reprod. Fertil. 4:175–188.

Perry, J. S., Heap, R. B. and Amoroso, E. C. (1973). Steroid hormone production by pig blastocysts. Nature 245:45–47.

Perry, J. S., Heap, R. B., Burton, R. D. and Gadsby, J. E. (1976). Endocrinology of the blastocyst and its role in the establishment of pregnancy. J. Reprod. Fertil. (Suppl.) 25:85–104.

Pietras, R. J. and Szego, C. M. (1975). Endometrial cell calcium and oestrogen action. Nature 253: 357–359.

Pietras, R. J. and Szego, C. M. (1977). Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature 265:69–72.

Rasmussen, H. and Goodman, D.B.P. (1977). Relationships between calcium and cyclic nucleotides in cell activation. Physiol. Rev. 57:421–509.

Roberts, R. M. and Bazer, F. W. (1980). The properties, function and hormonal control of synthesis of uteroferrin, the purple protein of the pig uterus. In: Steroid Induced Proteins (M. Beato, ed.). Elsevier/North Holland, Amsterdam, pp. 133–149.

Robertson, H. A. and King, G. J. (1974). Plasma concentrations of progesterone, estrone, estradiol-17β and estrone sulphate in the pig at implantation, during pregnancy and at parturition. J. Reprod. Fertil. 40:133–141.

Robertson, H. A., King, G. J. and Dyck, G. W. (1978). The appearance of estrone sulphate in the perIpheral plasma of the pig in early pregnancy. J. Reprod. Fertil. 52:337–338.

Rubin, R. P. and Laychock, S. G. (1978). Prostaglandins and calcium-membrane interactions in secretory glands. Ann. N.Y. Acad. Sci. 307:377–390.

Schlosnagle, D. C., Bazer, F. W., Tsibris, J.C.M. and Roberts, R. M. (1974). An iron-containing phosphatase induced by progesterone in the uterine fluids of pigs. J. Biol. Chem. 249:7574–7579.

Schlosnagle, D. C., Sanders, E. G., Bazer, F. W. and Roberts, R. M. (1976). The requirement of an essential thiol group and ferric iron for activity of the progesterone-induced porcine uterine purple phosphatase. J. Biol. Chem. 251:4680–4685.

Stoner, C. S., Geisert, R. D., Bazer, F. W. and Thatcher, W. W. (1981). Characterization of estrogen patterns in early pregnant and estrous gilts. Proceedings 73rd Annual Meeting American Society of Animal Science, Raleigh, NC, Abstr. #597.

Watson, J. and Patek, C. E. (1979). Steroid and prostaglandin secretion by the corpus luteum, endometrium and embryos of cyclic and pregnant gilts. J. Endocrinol. 82:425–428.

Zavy, M. T., Bazer, F. W., Thatcher, W. W. and Wilcox, C. J. (1980). A study of prostaglandin $F_2\alpha$ as the luteolysin in swine: V. Comparison of prostaglandin F, progestins, estrone and estradiol in uterine flushings from pregnant and nonpregnant gilts. Prostaglandins 20:837–851.